



Saskatchewan
Agriculture, Food
and Rural
Revitalization

ADF

AGRICULTURE DEVELOPMENT FUND

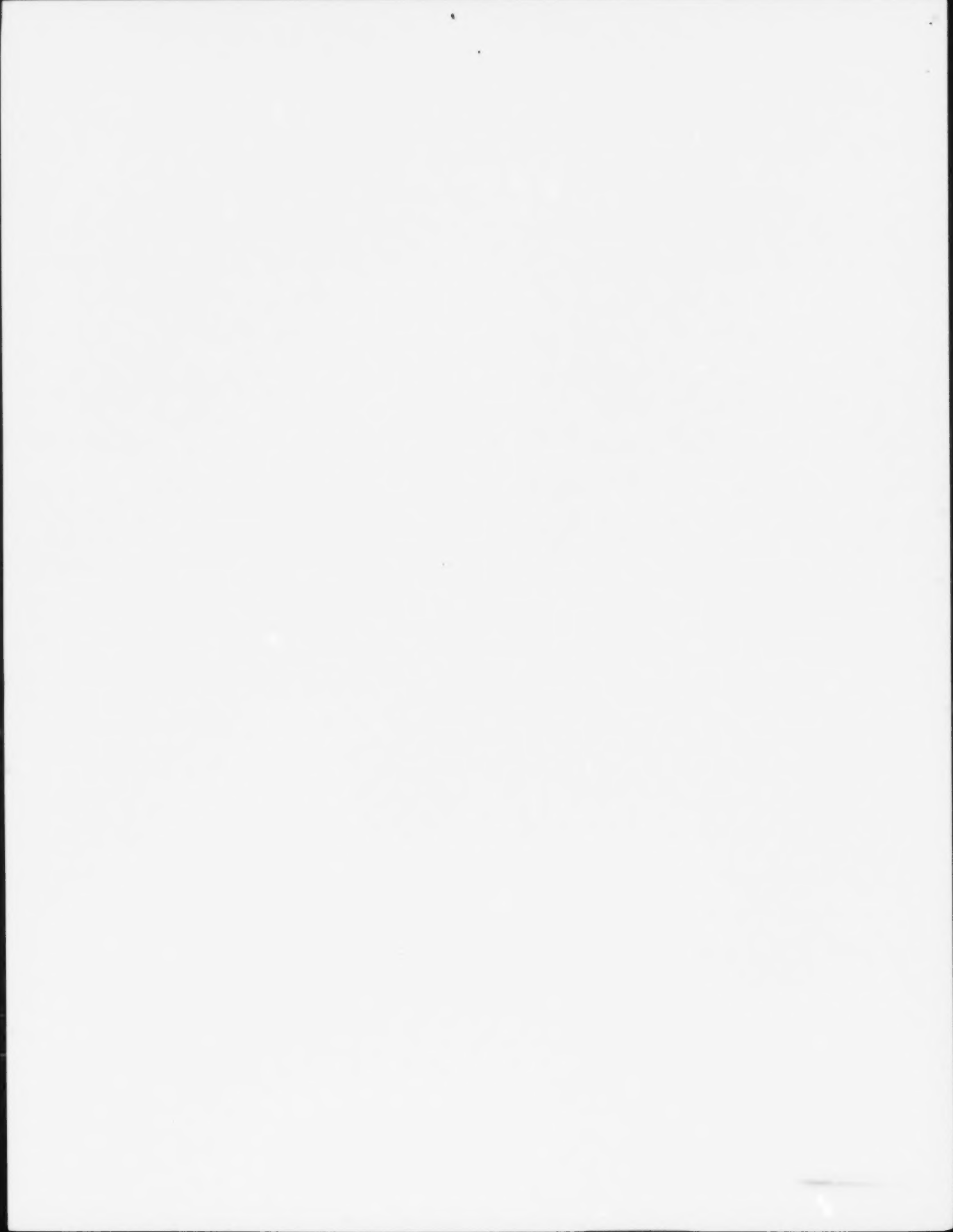
FINAL REPORT

19990246

ALGINATE MICROSPHERE DELIVERY SYSTEM
FOR ORAL IMMUNIZATION OF CATTLE

Funded by:
The Agriculture Development Fund

February 2004
Prepared by: VIDO



FINAL REPORT

ADF PROJECT #19990246

**"Alginate Microsphere Delivery System for Oral Immunization of
Cattle"**

Philip Griebel

**Veterinary Infectious Disease Organization
University of Saskatchewan
120-Veterinary Road
Saskatoon, Saskatchewan
S7N 5E3**

**Tel: 306-966-7472
Fax: 306-966-7478**

December, 2003

Table of Contents

	Page
List of Tables and Figures	3
Acknowledgements	3
Summary	4
Technical Report	5
Background	5
Objectives	7
Project Activities, Achievements, Publications	9
Results	10
Indications for Further Development	18
Information of Benefit to Producers	19
Personnel	19
Equipment	Not Applicable
Project Developed Materials	One Reprint
Acknowledgements of Support	One Manuscript (In Preparation)

LIST OF TABLES AND FIGURES

	Page
Figure 1 Release of soluble bovine IFN γ from bacteria	11
Figure 2 IFN γ released in vitro is biologically active	13
Figure 3 Encapsulated IFN γ is biologically active in cattle	14
Figure 4 Encapsulated IFN γ enhances vaccine responses	16
Figure 5 Encapsulated IFN γ qualitatively changes the vaccine response.	17

ACKNOWLEDGEMENTS

We thank Dr. Don Wilson and the Animal Care staff at VIDO for assistance with the clinical trials and the collection of samples. Financial support for this project was also provided by the Alberta Agricultural Research Institute, the Ontario Cattleman's Association, the Saskatchewan Health Services Utilisation and Research Commission, and DowAgro Sciences.

SUMMARY

The objective of this project was to develop an effective oral vaccine delivery technology that could be used to deliver a variety of vaccine antigens. An effective oral vaccine delivery system must prevent vaccine degradation during passage through the stomach and must ensure efficient targeting of the vaccine to the immune system (Peyer's patches) in the small intestine. Alginate microspheres were selected as the oral vaccine delivery technology that could be applied with a broad variety of vaccine antigens. The feasibility of using alginate microspheres for oral vaccine delivery was evaluated by 1) identifying microsphere formulations that provided the most efficient oral vaccine delivery and 2) evaluating the capacity of oral vaccines to prevent disease. Alginate microspheres were an effective delivery vehicle but were not practical for commercial vaccine production. Therefore, we investigated the use of bacterial encapsulation for oral delivery of a vaccine antigen (rotavirus P1P5 protein). This vaccine delivery system induced an immune response in young calves but a large amount of vaccine antigen was required to induce a relatively weak immune response. Therefore, in the final reporting period we investigated a novel methodology for enhancing immune responses to bacterial encapsulated antigen. Bacterial encapsulation of bovine interferon-gamma (IFN γ), a potent immune stimulatory molecule, was found to significantly enhance vaccine responses. We demonstrated that bacterial encapsulation was an effective approach for the efficient delivery of biologically active bovine IFN γ which significantly enhanced antibody responses to a vaccine antigen. Furthermore, the nature of these antibody responses supported the conclusion that bacterial encapsulated IFN γ could be used to enhance the immune protection induced by a vaccine antigen.

TECHNICAL REPORT

Background

The cattle industry has identified meat quality as a primary issue that may limit market share and consumer confidence. One factor that seriously affects the consumers' perception of meat quality is the presence of injection site lesions and needle fragments in the meat. Furthermore, due to carcass trim, injection site lesions have been estimated to cost the Canadian beef industry approximately \$9.00/head. **The objective of the present proposal was to develop an effective vaccine delivery system that could replace the use of needles for vaccination.** Producers have indicated that oral vaccines would address the issue of meat quality and would provide a cost-effective and labour efficient method of vaccination, especially if delivered through feed or water.

Oral vaccination of ruminants poses a number of challenges such as degradation of the vaccine by rumen flora and rapid clearance of the vaccine in the small intestine. Furthermore, it is important that the vaccine be delivered to the appropriate sites in the small intestine (Peyer's patches) to ensure efficient induction of mucosal immunity. Thus, a delivery system is required that ensures safe passage of vaccines through the rumen, that efficiently targets vaccines to the Peyer's patches, and that induces protective immune responses. One vaccine delivery technology that appeared capable of meeting these criteria was alginate microspheres.

Alginate is a very inexpensive compound and alginate micropsheres can be formulated without the use of organic solvents or high temperatures. The work of Dr. Terry Bowersock (Pharmacia-Upjohn) and work completed by our group at VIDO, during the last 3 years, has provided substantial evidence that alginate micropsheres can be used

to orally immunise cattle. To ensure that oral vaccine delivery technology is practical and cost-effective, however, further work was required to identify optimal vaccine formulations that achieve efficient vaccine passage through the rumen and efficient uptake by the Peyer's patches in the small intestine. Furthermore, these vaccine formulations must induce protective immune responses not only in the intestine and lungs (mucosal immunity) but ideally also throughout the rest of the body (systemic immunity).

Once we establish proof of principle for oral vaccination then it will be important to identify commercially viable oral vaccine delivery technologies. Alginate microspheres were investigated as one possible oral vaccine delivery technology that would be compatible with a variety of vaccine antigens. There were concerns, however, that large scale, commercial production of alginate microspheres would not be cost-effective. Therefore, we also investigated an alternative approach to encapsulate vaccine antigens for oral delivery. During the previous reporting period we demonstrated that bacterial encapsulation of a vaccine antigen (rotavirus P1P5) was an effective method for oral immunization of young calves. This approach was limited by the relatively large amount of vaccine antigen required to induce an immune response and the resulting immune responses were not as great as those observed following parenteral immunization. Therefore, during this reporting period we investigated a novel approach for enhancing immune responses to vaccine antigens encapsulated in bacteria. Bovine IFN γ , a potent immune stimulant, was also encapsulated within bacterial cells and was shown to effectively enhance immune responses to a vaccine antigen.

Objectives

The objective of this project was to identify a vaccine delivery system that could be used to orally immunize cattle with a variety of vaccine antigens. The selection of alginate microsphere formulations was based on two separate screening processes. First, we worked closely with Dr. Terry Bowersock (Pharmacia-Upjohn) to prepare a several microsphere formulations that were equivalent in their capacity to encapsulate vaccine antigens. These different formulations were screened at VIDO by using a unique animal model to compare multiple mucosal immune responses within the same animal. This screening process determined that the microsphere formulations efficiently delivered vaccine antigens to the Peyer's patches in the small intestine. This is a critical step in oral vaccine development since Peyer's patches are the most efficient site within the small intestine for the induction of immune responses.

Alginate microsphere formulations that performed well in Phase I of this project were evaluated for their capacity to prevent vaccine degradation in the rumen. The rumen contains a large number of microbes that effectively degrade ingested material. Alginate microspheres protected protein antigens against degradation in the rumen but the efficiency of vaccine delivery had not been previously evaluated with different formulations. Our evaluation of alginate microspheres also included discussions with animal health companies regarding the feasibility of using alginate microspheres to formulate commercial vaccines. These discussions revealed that the formulation process for antigen encapsulation in alginate microspheres would be very difficult to scale up to a commercial level. Therefore, in the next phase of the project we investigated alternative approaches for vaccine encapsulation.

Vaccine antigens have previously been expressed in bacteria but the vaccine antigens were then purified and used for intramuscular vaccination in traditional vaccine formulations. We re-evaluated this approach to vaccine production but from the perspective of oral vaccination. We investigated the possibility of using bacteria to both express and deliver oral vaccines. A commercial partner, Dow Agro Sciences, provided a patented technology that could be used to express large quantities of protein in bacteria. With this technology the bacteria were also processed to kill the bacteria and to enhance their stability as a delivery vehicle. Investigations during the previous reporting period demonstrated that bacteria could be used as a delivery vehicle for oral vaccination of calves but the oral vaccine induced weak immune responses. Therefore, the present reporting period addressed the final two objectives, which included formulating oral vaccines to optimise immune responses and disease protection.

Research objectives were achieved by completing the following milestones:

- | | |
|---|-------------|
| a) Optimise screening protocol for microsphere formulations (Complete) | July, 2000 |
| b) Identify microsphere formulations with consistent size (Complete) | Dec., 2000 |
| c) Evaluate efficiency of microsphere uptake by Peyer's patches (Complete) | July, 2001 |
| d) Vaccine delivery to Peyer's patches with optimal formulation (Complete) | April, 2002 |
| e) Formulate vaccine for oral delivery (Complete) | Aug., 2002 |
| f) Formulate oral vaccine to enhance immune responses (Complete) | Jan., 2003 |
| g) Evaluate immune protection induced by optimal formulation (Complete) | April, 2003 |

Project Activities, Achievements, Publications

A) Manuscripts

1. Mutwiri, G., Bowersock, T., Kidane, A., Sanchez, M., Gerdts, V., Babiuk, L.A. and P.J. Griebel. (2002). Induction of mucosal immune responses following enteric immunisation with antigen delivered in alginate microspheres. Vet. Immunol. Immunopath. 87: 269-276.
2. Kim, B., Bowersock, T., Griebel, P., Kidane, A., Babiuk, L.A., Sanchez, M., Attah-Poku, S., Kaushik, R.S., and G.K. Mutwiri. (2002). Mucosal immune responses following oral immunization with rotavirus antigens encapsulated in alginate microspheres. J. Controlled Release 85: 191-202.
3. Gaertner, F., Griebel, P., Webb, S., and Babiuk, L.A. Characterization of a novel bacterial expression system that can be used to enhance the biological activity of bovine cytokines. (Manuscript in Preparation).

B) Communications with Producer Organisations

Philip J. Griebel. Development of needle-free vaccines. Four oral presentations given to producers in Saskatoon area March 17-18, 2002.

C) Scientific Presentations

Mutwiri, G.K., Babiuk, L.A., Hecker, R., Thrush, T., Kaushik, R.S. and P.J. Griebel.

2002. CpG DNA enhances mucosal immune responses to an antigen delivered at a different mucosal site. 11th International Congress of Mucosal Immunology, Orlando, FL.

Feeding Immunity. 52nd Annual meeting of Canadian Society of Microbiologists. 2003.

June 18-20. Saskatoon, SK

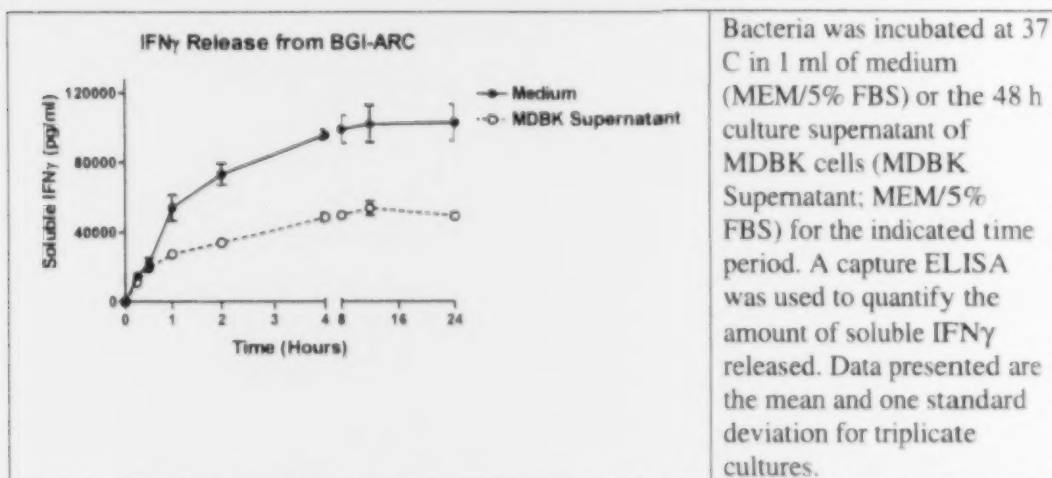
RESULTS

In the previous reporting period the primary objectives were to identify optimal formulation and delivery of a commercial vaccine antigen to Peyer's patches (April, 2002) and to then use this information to begin formulating a vaccine for oral delivery (Aug., 2002). The vaccine antigen selected for these studies was the rotavirus P1/P5 protein which contains the VP8 protein from rotavirus Type P1 ligated to the VP8 protein from Type P5 rotavirus. The 'intestinal-loop' model was used to determine the dose and formulation of soluble, purified P1P5 that could induce mucosal immune responses in the small intestine. Delivery efficiency was then compared between soluble protein and an *E. coli* bacterin containing insoluble P1P5 protein. With the intestinal "loop" model system we determined that the *E. coli* bacterin induced P1P5-specific mucosal immune responses within the small intestine. The bacterin, however, induced mucosal immune responses less efficiently than soluble P1P5 protein. Oral immunization of calves with the bacterin confirmed the results obtained with the intestinal "loop" model and thus the present reporting period (2003) addressed methods to enhance the immunogenicity and disease protection achieved with an oral bacterin.

The approach selected for the present studies was to design a bacterial delivery vehicle that expressed a potent immune stimulant to enhance immune responses to oral vaccines (Objective f- Jan. 2003). The molecule selected for these studies was bovine IFN γ since this cytokine had previously been shown to be an effective vaccine adjuvant in cattle. In these previous studies, soluble IFN γ was used to enhance vaccine responses but large quantities of cytokine were delivered by repeated injections, both before and after immunization. The first step in developing an oral bacterial delivery vehicle

containing bovine IFN γ was to determine if soluble IFN γ was released from the bacteria following treatment to stabilize the bacterial cells. *In vitro* assays determined that soluble IFN γ was gradually released from the bacterial cells over an 8-12 h period (Figure 1). This sustained release of IFN γ supported the conclusion that the bacterial cell delivery system would provide an appropriate delivery vehicle to ensure prolonged cytokine activity.

Figure 1. Release of soluble IFN γ from stabilized bacterial delivery vehicle.



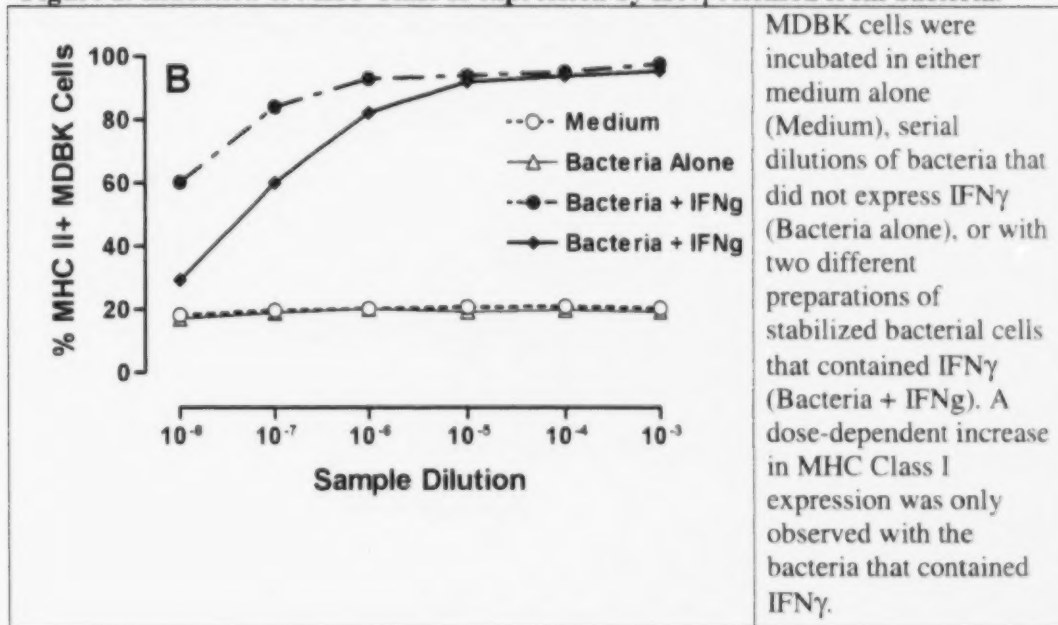
Conclusions

1. There is a progressive release of soluble IFN γ from the bacterial delivery vehicle and this release does not appear to be dependent upon proteolytic enzymes released from cultured MDBK cells.
2. Maximum release of IFN γ was observed within 4-8 hours and a significantly ($p < 0.001$) higher level of IFN γ was released in medium supplemented with 5% FBS (Medium).

3. A significantly ($p < 0.001$) lower amount of soluble IFN γ was detected when the bacterial delivery vehicle was incubated in culture supernatant of MDBK cells. This observation supports the conclusion that either less IFN γ was released in this medium or soluble IFN γ can be degraded following release from the bacterial delivery vehicle.

The next step in evaluating the new bacterial delivery system was to determine if the IFN γ being released was biologically active. This was determined using two different assays for well-defined biological activities associated with bovine IFN γ . We determined that IFN γ released from the bacterial delivery system had anti-viral activity and then investigated its capacity to induce major histocompatibility complex (MHC) class II expression on bovine cells since MHC class II protein plays a critical role in the induction of antigen-specific immune responses. These analysis revealed that the bacterial delivery system induced increased MHC class II expression only when bovine IFN γ was present within the delivery vehicle (Figure 2). These observations supported the conclusion that the soluble IFN γ released from the bacterial vehicle was biologically active.

Figure 2. Induction of MHC Class II expression by IFN γ released from bacteria.

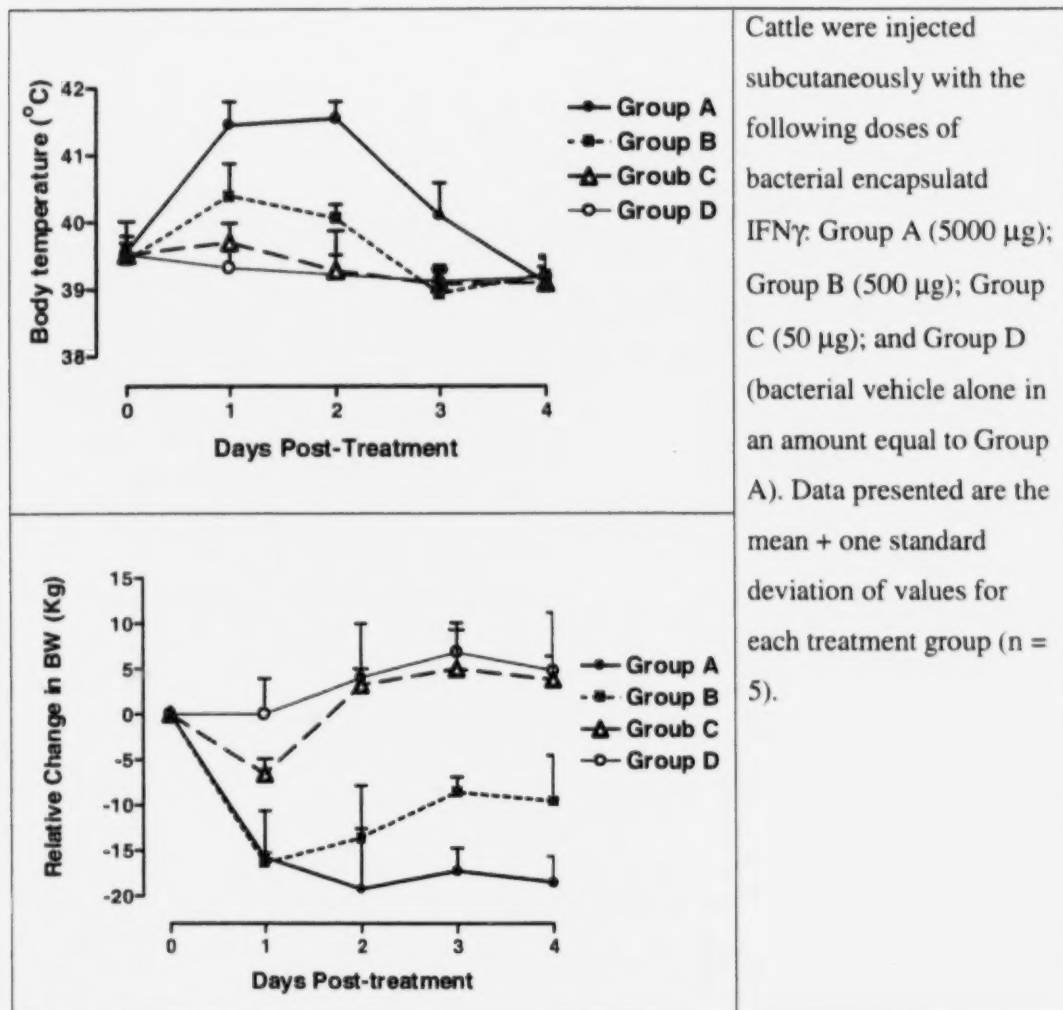


Conclusions

1. Cells incubated in the presence of the bacterial delivery vehicle containing IFN γ displayed a dose-dependent increase in MHC Class II expression.
2. The induction of increased MHC Class II supports the conclusion that IFN γ within the bacterial delivery vehicle was biologically active.

The biological activity of IFN γ was then confirmed by performing a dose titration trial in which cattle were injected subcutaneously with different doses of the bacterial delivery system. The safety of the bacterial delivery system was also evaluated by injecting cattle with the bacterial delivery system that either contained or lacked IFN γ cytokine expression. IFN γ is known to induce elevated body temperature (fever) and can induce a decrease in appetite that is associated with weight loss. Thus, these two parameters were monitored following the injection of the bacterial delivery system.

Figure 3. Bovine IFN γ encapsulated in the bacterial delivery system is biologically active in cattle.



The dose titration trial revealed that the injection of bacterial encapsulated bovine IFN γ resulted in a dose-dependent increase in body temperature and a reduction in body weight. These observations supported the conclusion that the bacterial delivery system could release biologically active IFN γ in the animal.

Conclusions:

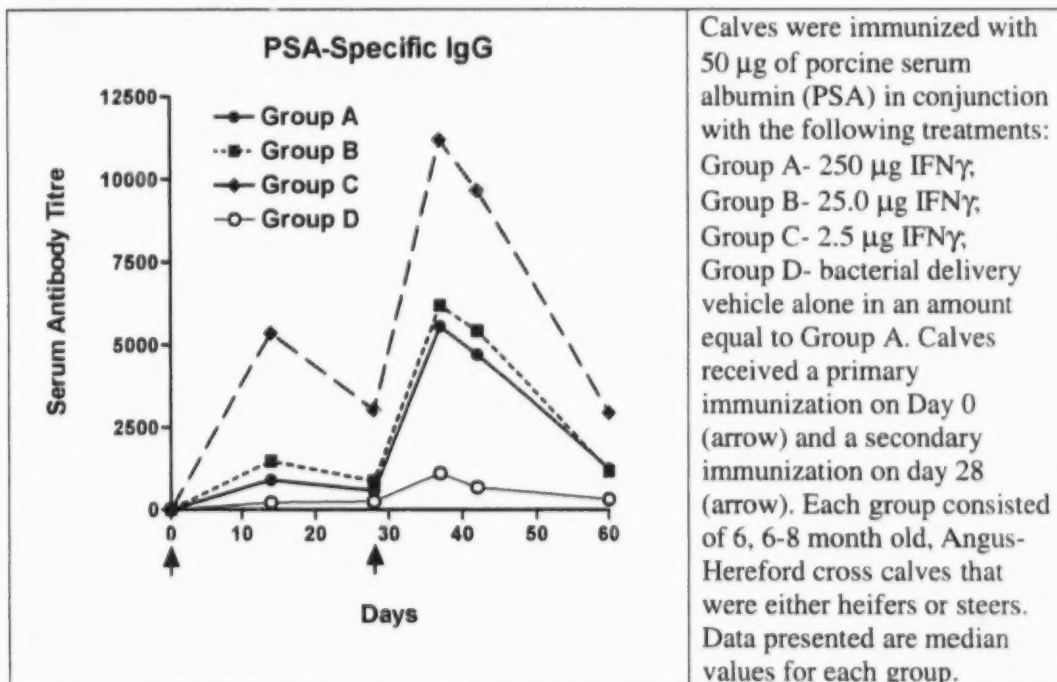
1. A single subcutaneous injection of approximately 5000 μg encapsulated IFN γ induced a significant elevation in body temperature for 48 h post-treatment and was associated with a prolonged (> 4 days) decrease in body weight.
2. A single subcutaneous injection of approximately 500 μg encapsulated IFN γ induced a mild elevation in body temperature ($< 1^\circ\text{C}$) for 48 h post-treatment and was associated with a prolonged (> 4 days) decrease in body weight.
3. A single subcutaneous injection of approximately 50 μg encapsulated IFN γ induced no detectable change in body temperature but was associated with a small (< 5 kg 4 days) decrease in body weight at 24 hour post-treatment.
4. A single subcutaneous injection of the bacterial delivery vehicle alone did not induce a detectable change in body temperature or body weight.

This clinical trial confirmed that bacterial encapsulated IFN γ was biologically in cattle but adverse clinical effects were observed with high doses of the cytokine. If this delivery system was to be used to enhance immune responses to an oral vaccine then adjuvant activity must occur with a dose of IFN γ that ensures no adverse clinical effects are associated with vaccination. Therefore, the adjuvant activity of bacterial encapsulated IFN γ was assessed in a dose titration trial. For this experiment we used a protein antigen (porcine serum albumin; PSA) for which we had established assays to measure immune responses. Furthermore, the doses of bacterial encapsulated IFN γ selected to test adjuvant activity spanned a range that fell below the dose of IFN γ that induced clinical effects in the previous trial. The adjuvant study revealed that bacterial encapsulated IFN γ significantly enhanced antibody responses in a dose-dependent manner (Figure 4). Both primary and secondary immune responses were enhanced and the most remarkable aspect of these observations is that the lowest dose of bacterial encapsulated IFN γ (2.5 μg) induced the greatest increase in antibody responses. This dose of IFN γ was over 10-fold less than the dose of bacterial encapsulated IFN γ that induced detectable clinical effects in the previous trial. Thus, these observations supported the conclusion that bacterial encapsulated IFN γ provided both an effective and safe approach to enhancing vaccine responses.

Conclusions

1. Bacterial encapsulated IFN γ functions as a vaccine adjuvant.
2. The adjuvant activity of IFN γ was dose-dependent but maximal activity was not associated with the highest dose of cytokine.
3. The dose of bacterial encapsulated IFN γ required to significantly enhance a vaccine response does not have a detectable clinical effect.

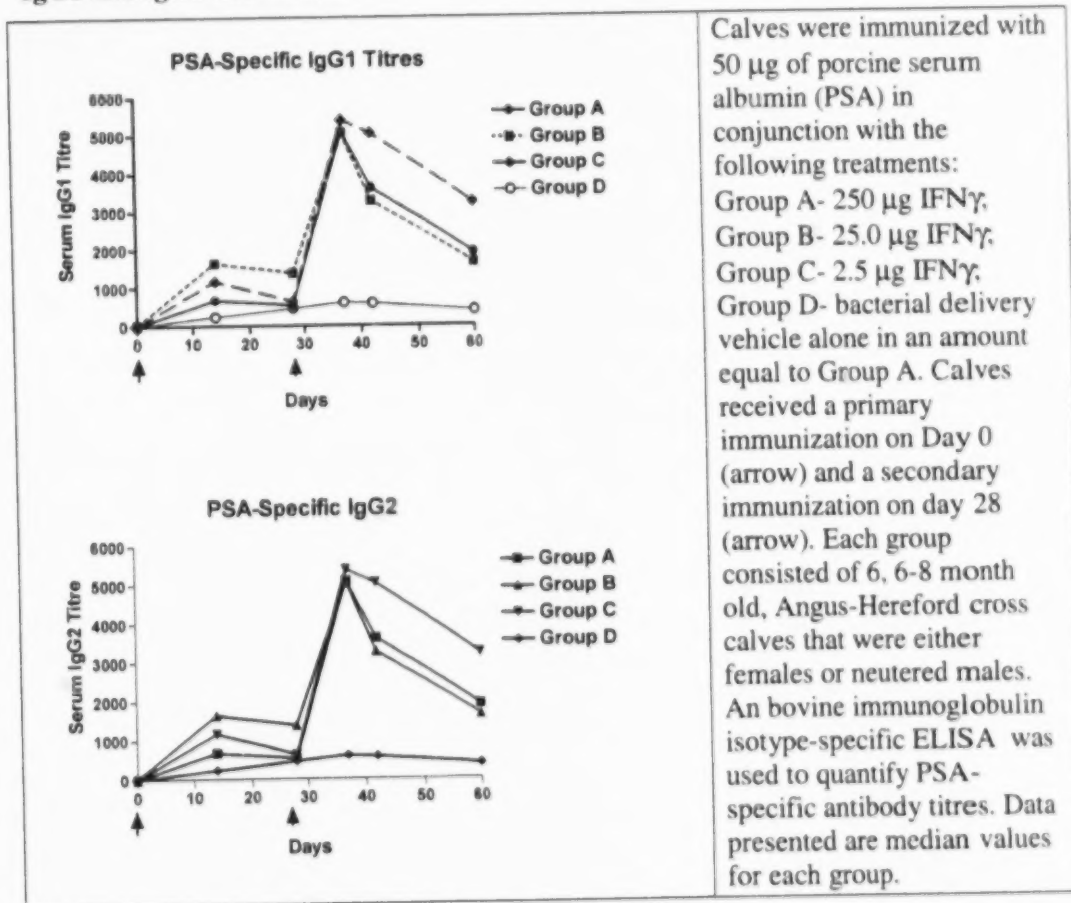
Figure 4. The adjuvant activity of bacterial encapsulated IFN γ .



After we observed that the bacterial encapsulated IFN γ functioned as an adjuvant then we investigated the possibility that this vaccine adjuvant might also enhance the immune protection induced by a vaccine. Previous investigations have confirmed that IFN γ is an important cytokine in the induction of immune responses that protect against viral infections. One aspect of these protective immune responses is the induction of antigen specific antibody of the IgG2 isotype since this type of antibody is more effective in viral neutralization. Therefore, we analysed the antibody isotype of the PSA-specific immune response (Figure 5). This analysis revealed that bacterial encapsulated IFN γ significantly

enhanced both the IgG1 and IgG2 PSA-specific antibody titres. Furthermore, the greatest increase for both antibody isotypes was again observed with the lowest dose of bacterial encapsulated IFN γ . Therefore, these observations support the conclusion that bacterial encapsulated IFN γ has the potential to significantly enhance disease protection.

Figure 5. Bacterial encapsulated IFN γ enhances the production of both PSA-specific IgG1 and IgG2 antibodies.



Conclusions:

1. Bacterial encapsulated IFN γ significantly increased both IgG1 and IgG2 PSA-specific antibody titres.
2. Maximal increase in antibody titres both isotypes was observed with the lowest dose (2.5 µg) of IFN γ .

Indications For Further Development

The present experiments confirmed that bacterial encapsulated IFN γ provided an effective delivery system for a cytokine that could significantly enhance immune responses to a vaccine antigen. Furthermore, dose titration studies revealed that this delivery system was very effective and the dose of encapsulated IFN γ required for adjuvant activity was 10-fold less than that required to induce a detectable clinical effect. Thus, this approach to vaccination should not have adverse effects that would have a negative impact on animal production. The capacity of this vaccine adjuvant system to alter immune responses both quantitatively and qualitatively supported the conclusion that this vaccine system has the capacity to significantly enhance the immune protection provided by vaccination. Further studies are now in progress to determine if this conclusion is correct and these studies are presently being supported by a commercial partner. The original objective of this research project was to design an effective oral vaccine delivery system for cattle and further studies are now required to determine if the bacterial encapsulated IFN γ significantly enhances both systemic and mucosal immune responses. Further funding has also been obtained to complete these studies and studies to determine the dose of bacterial encapsulated IFN γ required to enhance mucosal immune responses are in progress. Thus, the present investigation has led to the development of a novel vaccine delivery system. One of the most exciting aspects of this delivery system is that it can be effectively used to deliver both vaccine antigens and molecules to enhance immune responses to the vaccines.

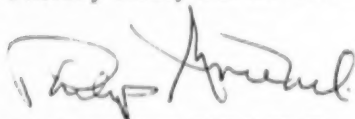
INFORMATION OF BENEFIT TO PRODUCERS

1. Significant progress has been made in identifying a vaccine delivery system for the oral immunization of cattle.
2. The safety of this new vaccine delivery system has been evaluated and no adverse clinical effects were observed.
3. A major barrier to oral immunization of cattle has been the failure to induce protective immune responses. The present investigations identified a molecule that significantly enhanced immune responses to vaccine antigens. The increased immune response was associated with an apparent increase in the capacity to protect against viral infections.
4. A commercial partner has now been identified to support the research required to determine if this new technology can be used with a commercial vaccine to enhance disease protection.

PERSONNEL

Dr. Philip Griebel	Research Scientist
Dr. George Mutwiri	Research Scientist
Dr. Radhey Kaushik	Research Associate
Dr. Don Wilson	Animal Care Veterinarian
Dr. Terry Bowersock	Alginate microsphere formulation (Pharmacia-Upjohn)
Dr. Steve Webb	Bacterial delivery system (Dow-Agro Sciences)
Terry Beskorwayne	Technician
Ponn Benjamin	Technician

I hereby certify that the above research has been completed.



Philip Griebel, DVM, Ph.D.

Senior Research Scientist

Mucosal immune responses following oral immunization with rotavirus antigens encapsulated in alginate microspheres

B. Kim^{a,b}, T. Bowersock^c, P. Griebel^a, A. Kidane^{c,1}, L.A. Babiuk^a, M. Sanchez^c,
S. Attah-Poku^a, R.S. Kaushik^a, G.K. Mutwiri^{a,*}

^aVeterinary Infectious Disease Organization, University of Saskatchewan, 120 Veterinary Road, Saskatoon, Saskatchewan, Canada S7N 5E3

^bNational Veterinary Research Institute, 480 Anyong, Kyunggi-Do, South Korea

^cPharmacia Animal Health, 7000 Portage Road, Kalamazoo, MI 49001-0199, USA

Received 12 December 2001; accepted 10 April 2002

Abstract

Availability of effective oral vaccine delivery vehicles should contribute to the success of oral immunization in domestic animals. To achieve this goal, we evaluated alginate microspheres for their capacity to induce mucosal immune responses following oral and enteric immunizations. Mice were immunized with either live porcine rotavirus (PRV) or its recombinant VP6 protein, encapsulated in alginate microspheres or unencapsulated. VP6-specific IgG (but no IgA) antibodies were detected in the sera of mice after a single intraperitoneal (i.p.) immunization with either VP6 in Incomplete Freund's adjuvant (VP6-IFA), VP6 in alginate microspheres (VP6-MS) or with live PRV in incomplete Freund's adjuvant (PRV-IFA). In contrast, VP6-specific IgA (but no IgG) was detected in culture supernatants of mesenteric lymph nodes from mice immunized i.p. with either VP6-IFA or with PRV-IFA. Oral immunization with VP6-MS induced the highest level of VP6-specific fecal IgA antibody, similar to responses induced by oral immunization with live PRV. Furthermore, the VP6-specific fecal IgA could be boosted by a secondary i.p. immunization with VP6. Further experiments were performed in a sheep intestinal 'loop' model to evaluate uptake of microspheres by Peyer's patches. Microspheres containing colloidal carbon were specifically bound and transported by follicle-associated epithelium of Peyer's patches. Additionally, mucosal immune responses were detected following enteric immunization with porcine serum albumin (PSA) encapsulated in alginate microspheres. Our results confirm that alginate microspheres are an effective oral delivery vehicle for protein antigens and intestinal IgA antibody responses are induced by antigens encapsulated in alginate microspheres without any additional mucosal adjuvant. These investigations confirm that alginate microspheres have the potential as an effective delivery vehicle for oral immunization of ruminants.

© 2002 Elsevier Science B.V. All rights reserved.

Keywords: Mucosal immunity; Oral vaccine delivery; Alginate microspheres

1. Introduction

Vaccination is the most cost-effective approach to prevent economic losses and morbidity caused by

*Corresponding author. Tel.: +1-306-96-67472; fax: +1-306-96-67478.

E-mail address: mutwiri@sask.usask.ca (G.K. Mutwiri).

¹Current address: Shire Laboratories, Rockville, MD, USA.

infectious diseases in animals. Most of the vaccines available today are injected parenterally and are not effective in inducing immunity at mucosal surfaces. Unfortunately, it is through these mucosal surfaces that the majority of pathogens invade the body. There is compelling evidence indicating that mucosal immunity is effectively induced when vaccines are delivered to the mucosa-associated lymphoid tissue (MALT) located at mucosal surfaces [1–3]. Thus, there is a need for effective mucosal vaccine delivery if better disease protection against mucosal pathogens is to be achieved.

Oral vaccination is the preferred route of immunization to induce mucosal immunity in the gastrointestinal tract, and has several advantages over other mucosal routes. Effectively delivered oral vaccines can induce mucosal, as well as systemic immunity [1,4]. Additionally, oral immunization uses a physiological delivery process (ingestion) that is non-invasive and eliminates needle injections which are frequently associated with tissue reactions that cost the beef industry up to \$9 per animal [5]. However, successful oral vaccination faces significant challenges due to the anatomic and physiologic barriers presented by the gastrointestinal tract. Oral administration of non-replicating vaccine antigens is usually associated with poor immune responses [1,6]. This is in part due to vaccines being degraded in the gastrointestinal tract before they reach the gut-associated lymphoid tissue (GALT), the inductive site for mucosal immune responses. Consequently, to achieve effective oral immunization, a 'protective' vehicle is required to deliver vaccine antigens to the GALT. Many such antigen delivery systems have been evaluated. Biodegradable microspheres have proven to be one of the most promising approaches [7,8]. 'M' (microfold) cells, located within the follicle-associated epithelium (FAE) overlying the Peyer's patches sample particulate antigens from the intestinal lumen. This uptake of particulate material appears to be size dependent. Microparticles with a diameter less than 10 μm are taken up by Peyer's patch M-cells [6,9]. Microspheres made from polylactide-L-glycolide (PLG) have been extensively investigated with various forms of antigens including protein and plasmid DNA [6,10]. While PLG microsphere formulations have successfully induced mucosal immune responses, their prepara-

tion can involve the use of organic solvents. These solvents, along with the internal pH of the degrading PLG microparticles may alter antigenic epitopes and hence compromise immunogenicity [11].

There are relatively few reports of using microspheres made from sodium alginate (kelp), a naturally occurring gelling polysaccharide that is extensively used in the food industry as a stabilizer and thickening agent. Sodium alginate is water soluble, and polymerizes into a solid matrix upon contact with divalent cations [12]. Procedures for formulating alginate microspheres are compatible with the use of a variety of antigens (proteins, live bacteria and viruses) for which immune responses have been demonstrated [4,13,14]. Additionally, alginate microspheres are stable at low pH [7], and can be formulated into a variety of particle sizes that can pass rapidly through the rumen of cattle [4,15].

Rotavirus infections are one of the major causes of diarrhea disease complex in young animals, including calves and pigs [16,17], and cause significant economic losses to the animal health industry [17]. Control of rotavirus-induced disease is presently achieved by passive immunization whereby pregnant females are immunized during the third trimester of pregnancy and maternal antibodies are transferred from mother to offspring through colostrum and milk [16]. While passive immunization has reduced rotavirus infection, newborn animals may fail to acquire adequate levels of maternal antibodies to achieve effective disease protection. This has led to a search for approaches to induce active immunity in the intestinal tract of young animals. Protection against rotavirus infection in various species has been shown to correlate with intestinal IgA [18,19]. Induction of active immunity in young animals would complement the passive immunity from mother's colostrum or milk. Unfortunately, active immunization of young animals is not extensively practiced, primarily due to two major concerns: (i) maternal antibody interference with vaccination [20–22]; and (ii) young animals are thought to have an immature immune system [20,21]. These concerns are supported by the observation that maternal antibodies can mediate high level of passive protection against rotavirus disease but active immune responses are suppressed in young pigs [23].

There may be effective ways, however, to induce

mucosal immune responses in the presence of maternal antibodies. Oral immunization of newborn mice with reovirus encapsulated in alginate microspheres induced intestinal IgA responses in the presence of maternal antibodies while unencapsulated virus did not [11]. This observation provided evidence that alginate microspheres could be used to circumvent the suppressive effects of maternal antibodies in vaccination of young animals. We recently reported that gut-associated lymphoid tissue (GALT) is immune competent in newborn ruminants and that maternal antibody did not interfere with enteric immunization [24], suggesting that oral immunization may be an effective approach in neonatal animals. Thus, by encapsulating rotavirus vaccine antigens in alginate microspheres, it would be possible to effectively vaccinate young animals and induce intestinal immunity even in the presence of maternal antibodies. This approach of a combined immunization program for mother and offspring should give maximum disease protection during the early days of life when young animals are most susceptible to disease.

The objective of this study was to evaluate whether porcine rotavirus antigens encapsulated in alginate microspheres could induce mucosal immune responses following oral immunization. Since the overall goal is to use alginate microspheres in ruminants, subsequent experiments were conducted in a sheep intestinal 'loop' model to evaluate uptake of microspheres by FAE of Peyer's patches and to determine if uptake of microsphere-encapsulated protein induced immune responses in GALT of ruminants.

2. Methods

2.1. Preparation of VP6 antigen

2.1.1. Cells and viruses

Porcine rotavirus strain 175 (Group A rotavirus) was isolated from piglets with diarrhea in South Korea. Rotavirus 175 was grown in MA-104 cells (provided by Professor Shien-Young Kang, Chungbuk National University). The recombinant baculovirus VP6:175 was propagated in confluent Sf-9 monolayers (ATCC, Manassas, VA) grown in Grace's

insect cell medium (Canadian Life Technologies, Burlington, ON) supplemented with 10% fetal bovine serum (Canadian Life Technologies).

2.1.2. Construction of recombinant baculovirus containing rotavirus VP6 gene cDNA insert

The rotavirus dsRNA was extracted from purified virus and denatured by methyl mercury hydroxide. The VP6 gene was reverse transcribed into cDNA and was amplified by RT-PCR [25,26]. The primers, VP6F1 and VP6R1 complementary to the 5' and 3' ends of VP6 gene were synthesized by DNA synthesizer (Applied Biosystems) on the basis of the sequence of Gottfried (Gene Bank accession number D00326) and restriction enzyme BamHI site was introduced into the primers for the convenience of the gene cloning into the plasmid vector. The sequence for the primer VP6F1 was

5'-CCG GAT CCG GCT TTT

AAA CAG AGT CTT C-3' BamHI

while the sequence for the second primer (VP6R1) was

5'-CCG GAT CCG GTC ACA

TCC TCT CAC TAT A-3' BamHI

The VP6 amplified gene was digested with restriction enzyme BamHI and cloned into the BamHI site of the transfer vector pVL1393 (Invitrogen Co). The construct, pVLVP6, placed the VP6 gene under the control of the strong AcNPV polyhedrin promoter. Recombinant baculovirus, VP6:175, was constructed by co-transfection of Sf-9 cells with the linearized baculovirus DNA and pVLVP6 using a Baculo Gold transfection Kit (PharMingen, San Diego, CA). The recombinant baculovirus was plaque-purified three times to eliminate contamination by wild-type baculovirus [27,28].

2.1.3. Detection and purification of VP6 protein

Baculovirus expressed VP6 was detected by an immunofluorescence assay. Sf-9 cells were infected with recombinant baculovirus VP6:175. After 3–5 days, the recombinant baculovirus infected cells grown on coverslip were fixed with cold acetone for 10 min at -20°C . VP6 specific monoclonal antibody or guinea pig hyperimmune 175 rotavirus antiserum

(National Veterinary Research and Quarantine Service, South Korea) were used to detect the expression of VP6.

VP6 protein was partially purified as follows: Sf-9 cells were infected with recombinant baculovirus. The infected cells were harvested by centrifugation ($1500 \times g$), resuspended in PBSA and washed two more times. Cells were lysed by sonication, centrifuged at $16\,000 \times g$ and the supernatant was used as antigen.

2.2. Encapsulation of antigens in alginate microspheres

The encapsulation of VP6 protein in alginate microspheres was done using the spray method described previously [4], with minor modifications. Briefly, VP6 protein or live virus was mixed in a 2% solution of medium viscosity sodium alginate (Sigma, St. Louis, MO, USA) to a final alginate concentration of 1.2%. The antigen–alginate mixture was sprayed under 40 p.s.i. of pressure through an ultrasonic nozzle (Turbosonic Inc., Waterloo, Ontario, Canada). Alginate droplets were collected into a beaker containing 2% CaCl_2 solution. The alginate cross-links with Ca^{2+} to form temporary microspheres, which were further stabilized by incubation with 0.2% poly-L-lysine (Sigma). The size of the microspheres was determined in a particle analyzer (Model 770 AccuSizer, Particle sizing Systems Inc., Santa Barbara, CA, USA), indicated that 80% of the microspheres had a diameter of 10 μm or less. To determine the efficiency of alginate encapsulation (loading efficiency), microspheres containing either the live virus or VP6 protein were lysed by incubation with calcium- and magnesium-free phosphate buffered saline (PBSA, pH 7.2). VP6 antigen was determined with the Bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). Virus titer was determined by plaque assay with MA-104 cells. Results from several experiments consistently showed that the encapsulation efficiency of both rotavirus protein and virus was approximately 30%. These loading efficiencies were used to determine the dose of antigen used for immunizations to ensure that equivalent amounts of antigen were used for encapsulated and unencapsulated antigen.

Colloidal carbon (India ink) was similarly en-

capsulated in alginate microspheres by the spray method. Following encapsulation, the microspheres were washed three times to remove free carbon particles.

Encapsulation of porcine serum albumin (PSA, Sigma) in alginate microspheres was done using a proprietary emulsion-cross-linking technique. Briefly, to encapsulate protein, the sodium alginate was mixed with PSA and an emulsion was prepared using a high pressure homogenizer (Emulsiflex® C5 homogenizer, Avestin Inc., Ottawa, CA, USA). Alginate droplets were cross-linked with a mixture of cations and the particle size was measured with a particle analyzer. The mean volume diameter for microspheres was 9–14 μm and greater than 90% of particles had a diameter less than 10 μm . To determine the loading efficiency of PSA, microspheres were lysed with $10 \times$ PBSA and the supernatant was then analyzed for total protein using the BCA protein assay (Pierce, Rockford, IL, USA).

2.3. Immunization of mice

An i.p. immunization experiment was conducted to confirm the immunogenicity of the rotavirus antigens in vivo, and to optimize the VP6-specific ELISA assay. BALB/c mice (Animal Resources Center, University of Saskatchewan) were immunized with a single i.p. injection with either of the following antigens: VP6 suspended in Incomplete Freund's adjuvant (VP6-IFA; $n=4$), VP6 encapsulated in alginate microspheres (VP6-MS; $n=4$) or with live porcine rotavirus in incomplete Freund's adjuvant (PRV-IFA; $n=4$). Control mice were injected with saline ($n=2$). Serum samples were collected prior to immunization and weekly after immunization. Mice were killed 4 weeks after immunization and mesenteric lymph nodes and Peyer's patch tissues were collected for immune assays.

For oral immunization experiments, 25 mice were allocated equally to five groups and each group was housed in a separate microfilter cage (Nalgene, Canada). Mice were orally immunized using a gavage needle (CDMV, Quebec, Canada) with a 4 week interval between each of the three oral immunizations. The dose of antigen used was 100 μg of VP6 protein and 50 μg of live PRV virus, either encapsulated in alginate microspheres or resuspended

in saline. A final i.p. immunization was done 4 weeks after the third oral immunization. Serum was collected on the day of each immunization and weekly after each immunization. Fecal samples were collected after the third immunization and weekly thereafter. To ensure fresh fecal samples were collected, mice were moved to clean cages with a wire-mesh floor and fecal pellets were collected 30 min later. Mice were euthanized 4 weeks after the final immunization at which time intestinal contents were collected from the colon.

2.4. Uptake of microspheres by GALT of sheep

To examine the interaction between microspheres and GALT, alginate microspheres were prepared and filtered through pores of different sizes to yield microspheres with the following approximate diameters: (i) less than 10 μm ($<10 \mu\text{m}$); (ii) greater than 10 μm but less than 20 μm ($10 \mu\text{m} < \text{MS} < 20 \mu\text{m}$); and (iii) greater than 20 μm ($>20 \mu\text{m}$). Microspheres of different sizes were injected into duplicate intestinal 'loops' containing a jejunal Peyer's patch (preparation of intestinal 'loops' is described below under Section 2.5). Intestinal tissue was collected 2 h after injection of microspheres, fixed in formalin and processed for routine histology. Tissue sections containing both FAE (overlying the PP) and mucosal villous epithelium were examined with the aid of a light microscope.

2.5. Immunization of sheep

Four month-old suffolk sheep were obtained from the Department of Animal and Poultry Science, University of Saskatchewan. Details of anesthesia and surgery for the preparation of intestinal 'loops' were described previously [13]. Briefly, a midline abdominal incision was made, a segment of jejunum containing eight consecutive jejunal PP was identified and transected at both ends ('intestinal-segment'). Continuity of the jejunum was re-established with an end-to-end anastomosis. The 'intestinal-segment' was flushed with PBSA to remove ingesta and an antibiotic solution (metronidazole, Abbot Laboratories, St. Laurent, PQ, Canada; enrofloxazine, Bayer Inc., Etobicoke, ON, Canada) was infused throughout the 'intestinal-segment'. The antibiotic

solution was removed by flushing with PBSA before both ends of the 'intestinal-segment' were closed. The 'intestinal-segment' was subdivided into eight 'loops' by tying silk ligatures 20 cm proximal and distal to each PP to create a space containing an individual PP ('loop'). Interspaces of various lengths separated each 'loop' containing a PP. All 'loops' and interspaces were injected with sodium-ampicillin (Novopharm, Toronto, ON, Canada). PSA antigen encapsulated in alginate microspheres was injected into individual 'loops'.

Duplicate 'loops' in each animal were injected with either 0.05, 0.1, 0.5 or 1.0 mg (per 'loop') of PSA encapsulated in alginate microspheres.

2.6. Organ and cell cultures

2.6.1. Mice

Organs from mice were cultured using a procedure described elsewhere [29], with minor modifications. Briefly, mesenteric lymph nodes (mesenteric LN) were removed from the abdomen and washed three times with PBSA containing antibiotics (penicillin, streptomycin and amphotericin B). Mesenteric LN were divided in half and each portion placed in one well of a 24-well culture plate containing 1 ml/well of serum-free AIM-V medium (GibcoBRL, Burlington, ON, Canada) supplemented with 2% FBS (GibcoBRL), 50 μM 2-mercaptoethanol (Sigma-Aldrich) and antibiotics. Peyer's patches were dissected out and similarly processed and cultured in AIM-V medium. Culture supernatants were collected from mesenteric LN, and PP after 4 days of culture, and stored at -20°C until assayed for VP6-specific antibody.

2.6.2. Sheep

Lambs were euthanized 3 weeks after immunization in intestinal 'loop'. PP were collected from each 'loop' and two control PP were collected from the normal jejunum adjacent to the site of anastomosis (outside the 'intestinal-segment' with 'loops'). The PP were dissected and placed separately in 10 ml ice-cold PBSA containing 0.1% ethylenediamine-tetraacetic acid (EDTA; BDH Inc., Toronto, ON, Canada). Intestinal contents were collected by cutting each 'loop' open along the mesenteric border and contents were added to PBSA at a 1:5 w/v ratio, and

stored at -70°C . To assay for secreted antibody, contents were thawed, particulate material was removed by centrifugation at $600\times g$ for 15 min and the supernatant used for immune assays. The PP cells were isolated as previously described [30]. Briefly, each PP was rinsed with ice-cold PBSA and placed in cold PBSA containing 0.1% EDTA. Lymphoid follicles and cells from the interfollicular region were released by mechanically separating the mucosa from the underlying muscularis externa and mucosal fragments were removed before preparing a single-cell suspension by repeatedly pipetting to disrupt lymphoid follicles. The resulting cell suspension was filtered through a $40\text{ }\mu\text{m}$ nylon mesh (Cell Strainer; Becton Dickinson Labware, NJ, USA), washed once in PBSA and resuspended in culture medium. Viable cells were identified by trypan blue exclusion and counted with a haemocytometer.

2.7. Antigen specific immune assays

2.7.1. Detection of VP6 specific antibodies in mice

VP6-specific serum IgG was detected as follows: 96 well microtiter plates (DYNEX Technologies, Inc., Chantilly, VA) were coated overnight with $0.5\text{ }\mu\text{g}$ per well of partially purified VP6 protein. The plates were washed with PBS containing 0.05% tween 20 (PBST). Serum samples were diluted 1:100 and added to wells. VP6-specific IgG or IgA was detected with alkaline phosphatase-conjugated goat anti-mouse IgG or IgA (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD), respectively. The color reaction was developed with *p*-nitrophenyl phosphate (PNPP) substrate (Sigma, St. Louis, MO). Absorbance was read at 405 nm wavelength on a BIO-RAD model 3550 microplate reader (BIO-RAD Laboratories, Hercules, CA), and reported as optical density (O.D.) above that of a negative control sample.

Feces and intestinal contents were treated with protease inhibitors [31] to prevent degradation of immunoglobulins by proteases before anti-VP6 antibodies were assayed. Samples were dissolved in cold PBSA at a ratio of 1:32 (w/v), vortexed vigorously, and any clumps were dispersed with a pipette. Samples were centrifuged, the pellet was discarded and the supernatant was further clarified by centrifugation. PMSF and sodium azide were added to

supernatants and incubated at room temperature for 15 min. Fetal bovine serum was added and the samples were stored at -20°C .

VP6-specific IgA in fecal samples was determined by ELISA. Microtiter plates were coated with VP6 protein and $100\text{ }\mu\text{l}$ of processed fecal samples were added to each well. Wells were washed and then incubated first with biotinylated goat anti-mouse IgA (Cedarlane Laboratories, Hornby, ON) and then with alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch Lab., Inc., Westgrove, PA). Alkaline phosphatase activity was detected with PNPP substrate (Sigma) and absorbance was read as described above and optical density (O.D.) was reported.

2.7.2. Detection of PSA-specific antibody secreting cells and secreted antibody in sheep intestine

PSA-specific antibody secreting cells (ASC) were detected using a modified ELISPOT assay described previously [13]. Briefly, microtiter nitrocellulose filtration plates (Whatman Inc., Clifton, NJ, USA) were coated with PSA ($500\text{ }\mu\text{g/ml}$) and 0.1×10^6 PP cells/well or 0.5×10^6 PP cells/well were added to triplicate wells. Following overnight culture, PSA-specific ASC were detected by first adding biotinylated rabbit anti-sheep IgG (H+L chain specific; 1:6000 dilution; Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA), followed by AP-conjugated streptavidin (1:1000 dilution; Jackson ImmunoResearch, Lab. Inc., Westgrove, PA, USA). ASC were visualised with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) insoluble alkaline phosphatase substrate (Sigma-Aldrich). The frequency of PSA-specific ASC per 1×10^6 cells was calculated by subtracting the number of spots present in wells not coated with PSA from the number of spots present in PSA-coated wells. An inverted light microscope was used to count four quadruplicate wells for each cell population and data presented are mean values.

2.8. Statistical analysis

Data (except pooled fecal samples) were analyzed by a Kruskal–Wallis one-way nonparametric analysis of variance using Statistix software (Analytical Soft-

ware, Tallahassee, FL). The level of significance was set at $P=0.05$.

3. Results

3.1. Mouse studies

An i.p. immunization experiment was conducted to confirm the immunogenicity of the recombinant rotavirus VP6 protein *in vivo*. VP6-specific IgG antibody responses were detected in sera of mice 4 weeks after a single i.p. immunization with VP6-IFA, VP6-MS and PRV-IFA, and antibody levels in the latter group were significantly elevated above saline controls (Fig. 1a). No VP6-specific IgA was detected in serum (data not shown). In contrast, VP6-specific IgA antibodies were detected in culture supernatants of mesenteric lymph nodes from mice immunized i.p. with VP6-IFA and PRV-IFA but not with VP6-MS (Fig. 1b). No VP6-specific IgG was detected in culture supernatants of mesenteric LN (data not shown) and no VP6-specific antibodies of either IgG or IgA isotype were detected in culture supernatants of PP (data not shown). These observations confirmed the immunogenicity of the VP6 vaccine antigen and indicated that vaccine formulation could change the immunogenicity of the VP6 protein.

After confirming the immunogenicity of the rotavirus antigens, we then evaluated whether oral immunization with rotavirus antigens formulated in alginate microspheres would induce systemic and mucosal immune responses. Antibody responses were then assayed in sera of orally immunized mice. Three oral immunizations induced elevated VP6-specific IgA antibody responses in pooled fecal samples from all groups of orally immunized mice compared to controls (Fig. 2a). However, no fecal IgG was detected (data not shown). Oral immunization did not induce any detectable serum VP6-specific antibodies (IgG or IgA) (data not shown). When orally immunized mice and the saline controls were injected i.p. with VP6-IFA, the i.p. immunization boosted mucosal immune responses as indicated by the higher IgA antibodies detected in feces collected from mice 10 days after the i.p. injection (Fig. 2b). Modest fecal IgA was detected in mice after the primary i.p. immunization of the control group,

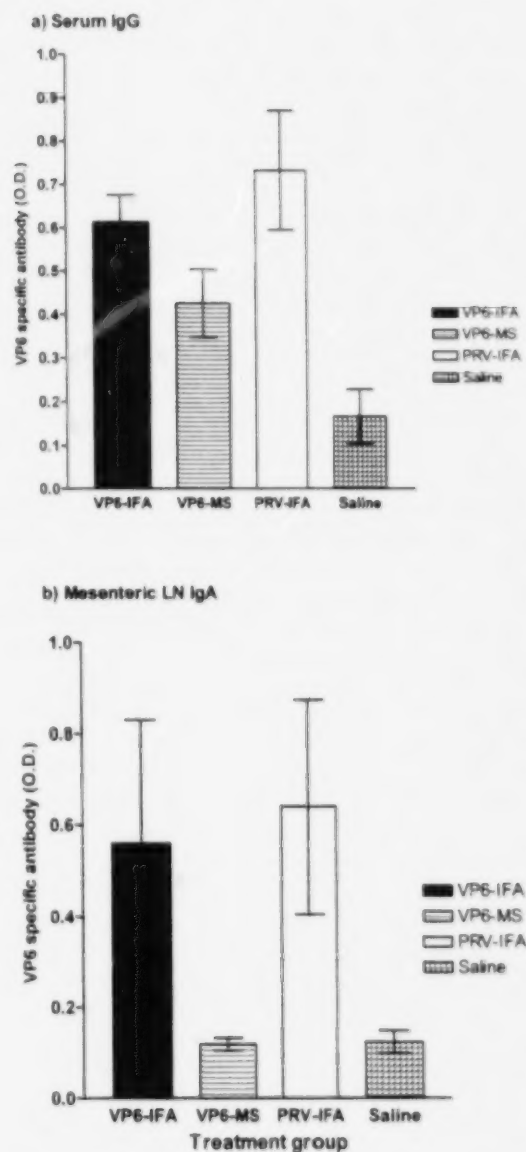


Fig. 1. VP6-specific antibody responses in (a) serum, and in (b) culture supernatants of mesenteric lymph nodes of mice 4 weeks after a single i.p. immunization with VP6 protein in incomplete Freund's adjuvant (VP6-IFA), VP6 in alginate microspheres (VP6-MS), PRV in incomplete Freund's adjuvant (PRV-IFA) or saline alone. Data presented are mean $\pm$ S.E.M. of values for each group.

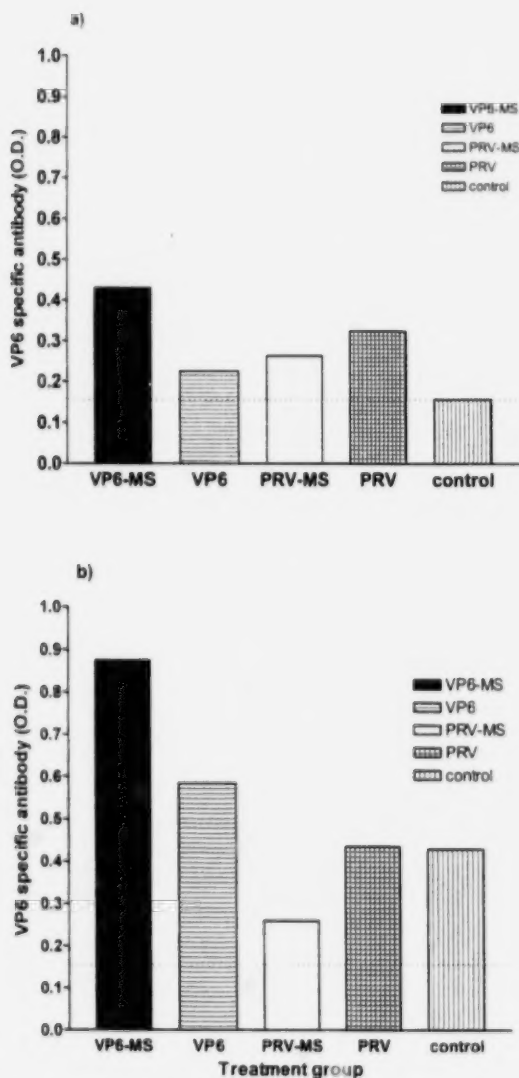


Fig. 2. VP6-specific IgA in feces of mice after (a) three oral immunizations and (b) after three oral followed by an i.p. immunization. Mice were orally immunized three times with either VP6 in alginate microspheres (VP6-MS), VP6 protein (VP6), porcine rotavirus (PRV) porcine rotavirus in alginate microspheres (PRV-MS), PRV in saline or saline alone (control). An i.p. booster immunization was done with VP6 protein in IFA. Data presented are O.D. for pooled fecal samples (one sample per group, hence no error bars). Dotted line indicates background O.D. from fecal samples of non-immunized control mice (Fig. 2a) analyzed within the same assay.

confirming our earlier observation that a single i.p. immunization can induce an intestinal IgA response.

Results from the pooled fecal samples were confirmed when intestinal contents collected from individual mice 18 days after the i.p. injection were assayed for antibody responses. VP6-specific IgA antibody responses were detected in the intestinal contents of mice in all groups (Fig. 3). The lower IgA responses detected in intestinal contents from individual mice (Fig. 3) compared to pooled fecal samples (Fig. 2b) may be due the fact that the former samples were collected 8 days after that the latter when IgA levels may have been declining. The VP6-MS group had the highest IgA antibody response, but was not significantly different from non-encapsulated VP6.

3.2. Sheep studies

The efficacy of microsphere formulations is thought to be due to the increased uptake by M cells. Therefore, the interaction between alginate microspheres and FAE was investigated. Microspheres containing colloidal carbon were attached only to the FAE, the epithelium overlying the PP follicles, but

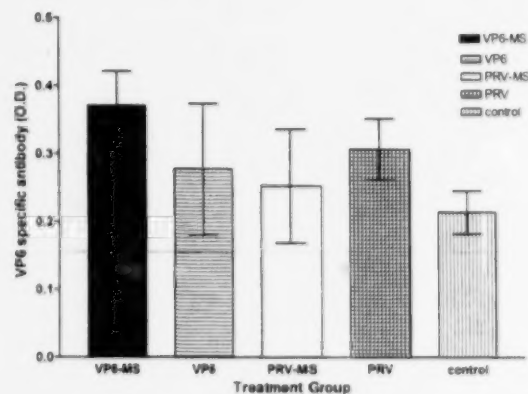


Fig. 3. VP6-specific IgA in intestinal contents of mice after three oral followed by one i.p. immunization. Mice were orally immunized three times with either free VP6 in alginate microspheres (VP6-MS), VP6 (VP6), porcine rotavirus in alginate microspheres (PRV-MS), PRV in saline or saline alone (control). i.p. booster immunization was done with VP6 protein in IFA. Data presented are mean $\pm$ S.E.M. of values for each group. Dotted line indicates background O.D. from fecal samples of non-immunized control mice (Fig. 2a) analyzed within the same assay.

were not attached to the villous epithelium in the non-Peyer's patch areas (Fig. 4). Additionally, microspheres attached to the FAE only in sections from loops injected with microspheres $<10\text{ }\mu\text{m}$ but

not in those from 'loops' injected with larger microspheres ($10\text{--}20\text{ }\mu\text{m}$ and $>20\text{ }\mu\text{m}$). Furthermore, following a 2 h period, colloidal carbon was visible within the lymphoid follicles of the PP (Fig. 4). The

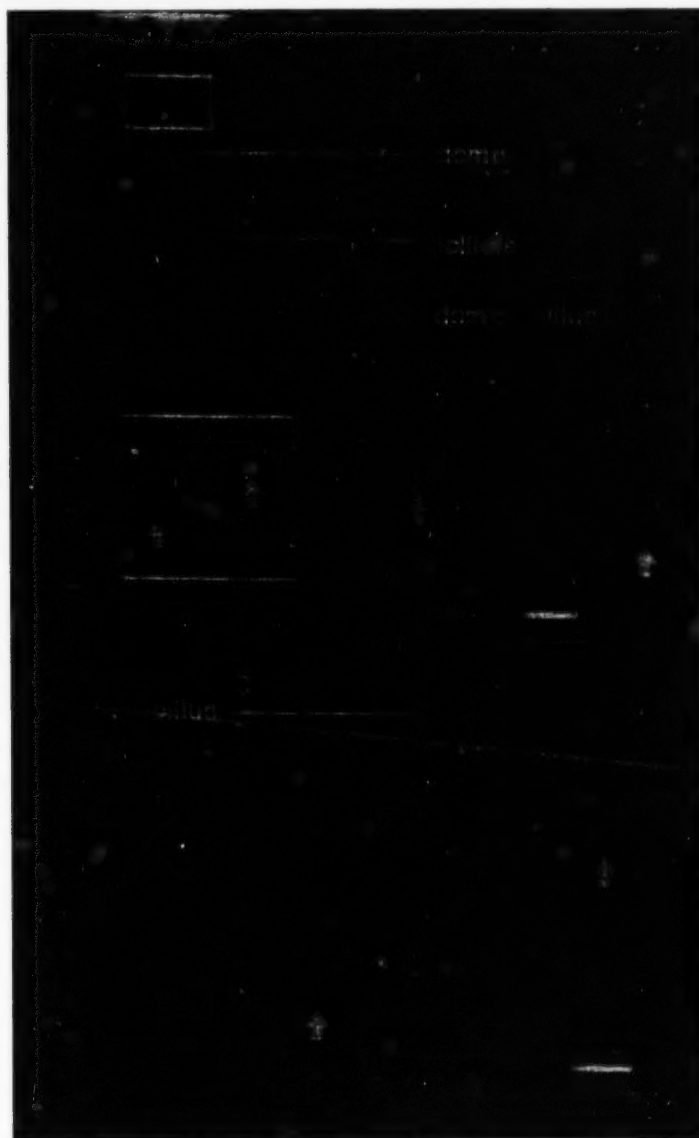


Fig. 4. Uptake of algininate microspheres by GALT of ruminants. Colloidal carbon was encapsulated in algininate microspheres and injected into intestinal 'loops' of sheep containing jejunal PP. Tissue sections were examined under a light microscope. Algininate microspheres containing colloidal carbon (arrows) were seen attached to the follicle-associated epithelium and in follicles of jejunal Peyer's patches.

were not attached to the villous epithelium in the non-Peyer's patch areas (Fig. 4). Additionally, microspheres attached to the FAE only in sections from loops injected with microspheres $<10\text{ }\mu\text{m}$ but

not in those from 'loops' injected with larger microspheres ($10\text{--}20\text{ }\mu\text{m}$ and $>20\text{ }\mu\text{m}$). Furthermore, following a 2 h period, colloidal carbon was visible within the lymphoid follicles of the PP (Fig. 4). The

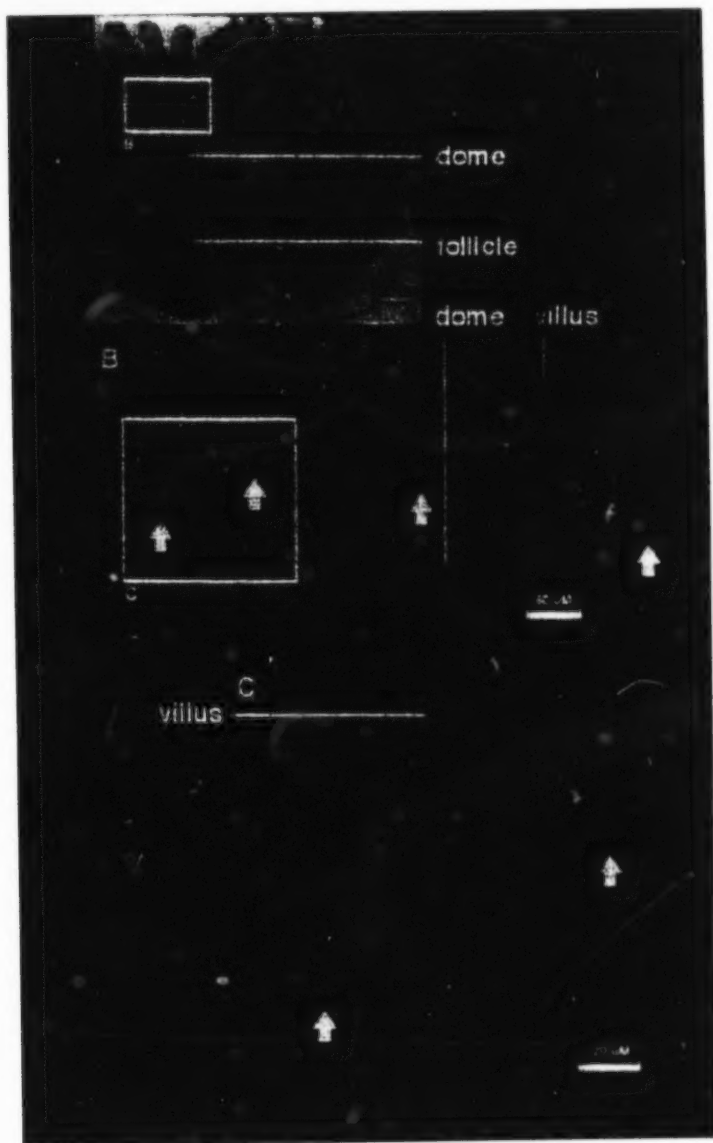


Fig. 4. Uptake of alginate microspheres by GALT of ruminants. Colloidal carbon was encapsulated in alginate microspheres and injected into intestinal 'loops' of sheep containing jejunal PP. Tissue sections were examined under a light microscope. Alginate microspheres containing colloidal carbon (arrows) were seen attached to the follicle-associated epithelium and in follicles of jejunal Peyer's patches.

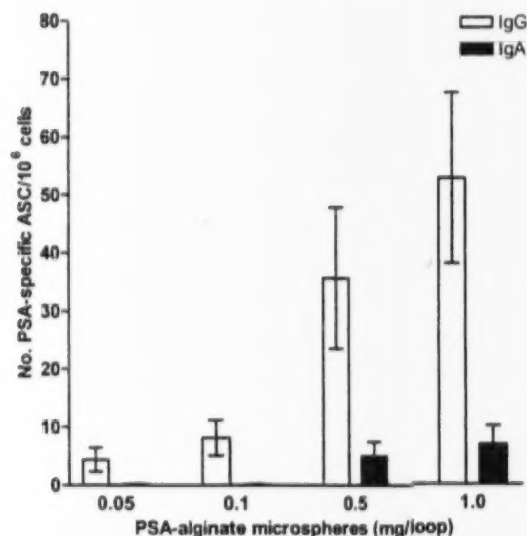


Fig. 5. Frequency of PSA-specific antibody secreting cells (IgG and IgA) in Peyer's patches of sheep. Intestinal 'loops', each containing a jejunal PP were injected with PSA encapsulated in alginate microspheres. Immune responses were assayed by ELISPOT assay 3 weeks after a single immunization and data presented are means ($\pm$ S.E.M.) of ASC counted from four 'loops'.

uptake of microspheres was also confirmed by the presence of numerous PSA-specific antibody secreting cells (ASC) of IgG and IgA (Fig. 5) isotypes collected from 'loops' injected with PSA-MS. The number of ASC within the PP also correlated with the dose of encapsulated antigen injected into each intestinal 'loop'. No ASC cells were detected in control PP collected from the normal adjacent jejunum (jejunum outside the 'intestinal-segment' with the 'loops') of animals with immunized intestinal 'loops'.

4. Discussion

The encapsulation of antigens in microspheres is thought to reduce antigen degradation and enhance antigen delivery to GALT [1,7]. The present investigation confirmed that encapsulating PRV and its VP6 protein in alginate microspheres effectively induced antigen specific IgA antibody responses in

the intestinal tract following oral immunization of mice. Alginate-encapsulated recombinant protein appeared to induce a slightly higher intestinal IgA response compared to unencapsulated VP6 protein. The uptake of microspheres in the intestinal tract is a size dependent phenomenon. Microparticles with a diameter of less than 10 μ m were taken up by 'M' cells in the Peyer's patches of mice and pigs, respectively [6,9]. The present investigation confirms that in the GALT of ruminants, as in the other species, microspheres <10 μ m are taken up by FAE within which 'M' cells are contained. Most importantly, colloidal carbon encapsulated in alginate microspheres was visualized in the follicles of PP where induction of immune responses occurs. These observations indicate that alginate microspheres 'target' PP (and presumably 'M' cells) and deliver encapsulated material to the intestinal immune system. In subsequent experiments we demonstrated that the uptake of microspheres was followed by successful induction of immune responses as indicated by the detection of PSA-specific antibody secreting cells in PP.

The pattern of redistribution of microspheres is also dependent on their size. Eldridge and coworkers [6] showed that microspheres within the range of 5–10 μ m were taken up by Peyer's patches and remained in the dome area, while those <5 μ m were detected in the Peyer's patches, mesenteric lymph nodes and spleen. This suggests that particle size may determine the type of the immune response elicited by vaccine-containing microspheres administered by the oral route. In this regard, microspheres less than 5 μ m would be predicted to induce a predominantly circulating antibody response based on their propensity to disseminate to systemic lymphoid tissue. In contrast, microspheres greater than 5 μ m would be predicted to raise predominantly a mucosal immune response because they remain in the IgA inductive environment of the Peyer's patches over the course of antigen release [6]. Thus, based on the size characteristics of the microspheres made using our procedure, we could speculate that there were relatively more microspheres in the 5–10 μ m diameter range, or microspheres of this size contained a sufficient amount of VP6 antigen to induce a detectable mucosal IgA response.

i.p. immunization induced a primary intestinal IgA

response in the group of mice that had not been previously immunized orally ('control'). Additionally, the i.p. immunization enhanced enteric IgA responses induced by prior oral vaccination. Recent reports have confirmed that the mesenteric lymph nodes are an alternate site for the induction of intestinal IgA responses in the absence of Peyer's patches [32]. In this regard, it is likely that i.p. injection of native protein or PRV drains to the mesenteric lymph node, thereby inducing IgA responses in the mesenteric LN. Effector lymphocytes would then disseminate to the intestinal tract as indicated by the detection of IgA in intestinal contents. No IgA antibodies were detected in culture supernatants of PP mice immunized i.p. with the rotavirus antigens. This is not surprising since effector cells such as IgA plasma cells disseminate primarily to the lamina propria from where they secrete the IgA. The IgA in turn is transported across the intestinal wall into the lumen by a receptor-mediated process. However, when the antigen is given orally, it is taken up from the lumen across the intestinal wall into the Peyer's patches, where induction of immune responses occurs, leading to generation of effector cells and the presence of IgA in feces. The booster effect of i.p. immunization in mice previously immunized orally suggests cross-communication between systemic and mucosal immune systems. Thus, immune cells stimulated by i.p. immunization support immune responses in the PP.

The procedure we used for making alginate microspheres is more likely to allow for retention of epitopes necessary for the induction of humoral immune responses than procedures requiring organic solvents [11]. In several experiments, infectious virus was recovered from lysed microspheres (unpublished observation). We speculate that encapsulation may have prevented the interaction between PRV and enterocytes. It is not clear why encapsulation of live PRV did not enhance immune responses in the same way as encapsulation of VP6 protein. It is possible that either the formulation or the coating of microspheres with poly-L-lysine stabilized the particles too much reducing the rate of release of the virus in vivo.

In summary, results from these studies confirm that alginate microspheres are an effective delivery system for the induction of IgG and IgA responses in

the intestinal tract following oral and enteric administration.

Acknowledgements

We thank Drs D. Godson, M. Baca-Estrada for their advice, Dr H. Townsend for help with the statistical analysis and Dr D. Wilson for help with the surgery model. We also thank B. Carrol, Donna Dent, and C. Olson for their technical assistance. Financial support for this work was provided by Saskatchewan HSURC, Natural Sciences and Engineering Research Council and Western CARDS. Dr L.A. Babiuk is a holder of the Canada Research chair in vaccinology. Published with permission of the director of VIDO as VIDO journal series no. 311.

References

- [1] W. Muir, A.J. Husband, E.M. Gipps, M.P. Bradley, Induction of specific IgA responses in rats after oral vaccination with biodegradable microspheres containing a recombinant protein, *Immunol. Lett.* 42 (3) (1994) 203–207.
- [2] A.M. Mowat, J.L. Viney, The anatomical basis of intestinal immunity, *Immunol. Rev.* 156 (1997) 145–166.
- [3] Z.Q. Xiang, S. Pasquini, H.C. Ertl, Induction of genital immunity by DNA priming and intranasal booster immunization with a replication-defective adenoviral recombinant, *J. Immunol.* 162 (11) (1999) 6716–6723.
- [4] T.L. Bowersock, H. HogenEsch, S. Torregrosa, D. Borie, B. Wang, H. Park, K. Park, Induction of pulmonary immunity in cattle by oral administration of ovalbumin in alginate microspheres, *Immunol. Lett.* 60 (1) (1998) 37–43.
- [5] J. Van Donkersgoed, S. Dixon, G. Brand, M. VanderKop, A survey of injection site lesions in fed cattle in Canada, *Can. Vet. J.* 38 (12) (1997) 767–772.
- [6] J.H. Eldrige, C.J. Hammond, J.A. Meulbroek, J.K. Staas, R.M. Gilley, T.R. Tice, Controlled release in the gut associated lymphoid tissue. I. Orally administered biodegradable microspheres target the Peyer's patches, *J. Controlled Release* 11 (1990) 205.
- [7] P.A. Offit, C.A. Khoury, C.A. Moser, H.F. Clark, J.E. Kim, T.J. Speaker, Enhancement of rotavirus immunogenicity by microencapsulation, *Virology* 203 (1) (1994) 134–143.
- [8] S. Raychaudhuri, K.L. Rock, Fully mobilizing host defense: building better vaccines, *Nat. Biotechnol.* 16 (11) (1998) 1025–1031.
- [9] R. Beier, A. Gebert, Kinetics of particle uptake in the domes of Peyer's patches, *Am. J. Physiol.* 275 (1 Pt. 1) (1998) G130–G137.
- [10] S.C. Chen, D.H. Jones, E.F. Fynan, G.H. Farrar, J.C. Clegg,

- H.B. Greenberg, J.E. Herrmann, Protective immunity induced by oral immunization with a rotavirus DNA vaccine encapsulated in microparticles, *J. Virol.* 72 (7) (1998) 5757–5761.
- [11] S.B. Periwai, T.J. Speaker, J.J. Cebra, Orally administered microencapsulated reovirus can bypass suckled, neutralizing maternal antibody that inhibits active immunization of neonates, *J. Virol.* 71 (4) (1997) 2844–2850.
- [12] T.L. Bowersock, S. Martin, Vaccine delivery to animals, *Adv. Drug Deliv. Rev.* 38 (2) (1999) 167–194.
- [13] V. Gerdis, R.R. Uwiera, G.K. Mutwiri, D.J. Wilson, T. Bowersock, A. Kidane, L.A. Babiuk, P.J. Griebel, Multiple intestinal 'loops' provide an in vivo model to analyse multiple mucosal immune responses, *J. Immunol. Methods* 256 (1–2) (2001) 19–33.
- [14] S.K. Mittal, N. Aggarwal, G. Sailaja, A. van Olphen, H. HogenEsch, A. North, J. Hays, S. Moffatt, Immunization with DNA, adenovirus or both in biodegradable alginate microspheres: effect of route of inoculation on immune response, *Vaccine* 19 (2–3) (2000) 253–263.
- [15] T. Bowersock, W.S.W. Shalaby, M. Levy, W.E. Blevins, M.R. White, D.L. Borie, K. Park, The potential use of poly-(methacrylic acid) hydrogels for oral administration of drugs and vaccines to ruminants, *J. Controlled Release* 31 (1994) 245–254.
- [16] L.J. Saif, F.M. Fernandez, Group A rotavirus veterinary vaccines, *J. Infect. Dis.* 174 (Suppl. 1) (1996) S98–S106.
- [17] J. Lee, L.A. Babiuk, R. Harland, E. Gibbons, Y. Elazhary, D. Yoo, Immunological response to recombinant VP8* subunit protein of bovine rotavirus in pregnant cattle, *J. Gen. Virol.* 76 (Pt. 10) (1995) 2477–2483.
- [18] S.E. Coffin, Induction of virus-specific antibody production by lamina propria lymphocytes following intramuscular inoculation using rotavirus, *J. Infect. Dis.* 172 (1995) 874.
- [19] D.O. Matson, Fecal antibody responses to symptomatic and asymptomatic rotavirus infections, *J. Infect. Dis.* 167 (1993) 577.
- [20] C.A. Siegrist, M. Cordova, C. Brandt, C. Barrios, M. Berney, C. Tougne, J. Kovarik, P.H. Lambert, Determinants of infant responses to vaccines in presence of maternal antibodies, *Vaccine* 16 (14–15) (1998) 1409–1414.
- [21] D.E. Hassett, J. Zhang, J.L. Whitton, Neonatal DNA immunization with a plasmid encoding an internal viral protein is effective in the presence of maternal antibodies and protects against subsequent viral challenge, *J. Virol.* 71 (10) (1997) 7881–7888.
- [22] G. Chappuis, Neonatal immunity and immunisation in early age: lessons from veterinary medicine, *Vaccine* 16 (14–15) (1998) 1468–1472.
- [23] D.C. Hodgins, S.Y. Kang, L. deArriba, V. Parreno, L.A. Ward, L. Yuan, T. To, L.J. Saif, Effects of maternal antibodies on protection and development of antibody responses to human rotavirus in gnotobiotic pigs, *J. Virol.* 73 (1) (1999) 186–197.
- [24] G. Mutwiri, C. Bateman, M.E. Baca-Estrada, M. Snider, P. Griebel, Induction of immune responses in newborn lambs following enteric immunization with a human adenovirus vaccine vector, *Vaccine* 19 (9–10) (2000) 1284–1293.
- [25] C.A. Dangler, C.A. de Mattos, C.C. de Mattos, B.I. Osburn, Identifying bluetongue virus ribonucleic acid sequences by the polymerase chain reaction, *J. Virol. Methods* 28 (3) (1990) 281–292.
- [26] M. Gorziglia, Y. Hoshino, K. Nishikawa, W.L. Maloy, R.W. Jones, A.Z. Kapikian, R.M. Chanock, Comparative sequence analysis of the genomic segment 6 of four rotaviruses each with a different subgroup specificity, *J. Gen. Virol.* 69 (Pt. 7) (1988) 1659–1669.
- [27] M.K. Estes, S.E. Crawford, M.E. Penaranda, B.L. Petrie, J.W. Burns, W.K. Chan, B. Ericson, G.E. Smith, M.D. Summers, Synthesis and immunogenicity of the rotavirus major capsid antigen using a baculovirus expression system, *J. Virol.* 61 (5) (1987) 1488–1494.
- [28] D.A. Lindsay, S.L. Vonderfecht, M.J. Betenbaugh, J.J. Eiden, Baculovirus expression of gene 6 of the IDIR strain of group B rotavirus (GBR): coding assignment of the major inner capsid protein, *Virology* 193 (1) (1993) 367–375.
- [29] K.A. Brown, C.A. Moser, T.J. Speaker, C.A. Khoury, J.E. Kim, P.A. Offit, Enhancement by microencapsulation of rotavirus-specific intestinal immune responses in mice assessed by enzyme-linked immunospot assay and intestinal fragment culture, *J. Infect. Dis.* 171 (5) (1995) 1334–1338.
- [30] P. Griebel, Isolation of lymphoid follicles from Peyer's patches, in: I. Lefkowitz (Ed.), *Immunology Methods Manual*, Vol. 3, Academic Press, London, 1997, p. 2079.
- [31] C.O. Elson, W. Ealding, J. Lefkowitz, A lavage technique allowing repeated measurement of IgA antibody in mouse intestinal secretions, *J. Immunol. Methods* 67 (1) (1984) 101–108.
- [32] M. Yamamoto, P. Rennert, J.R. McGhee, M.N. Kweon, S. Yamamoto, T. Dohi, S. Otake, H. Bluethmann, K. Fujihashi, H. Kiyono, Alternate mucosal immune system: organized Peyer's patches are not required for IgA responses in the gastrointestinal tract, *J. Immunol.* 164 (10) (2000) 5184–5191.